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Antibacterial, bacteriolytic, antibiofilm, and synergistic effects of *Curcuma* species ethanol extracts with antibiotic against multidrug resistant *Pseudomonas aeruginosa*

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ABSTRACT

Introduction: *Curcuma* species have shown antibacterial activity against *Pseudomonas aeruginosa*. The current study was conducted to analyze the antibacterial activity of ethanol extracts of *Curcuma* species rhizomes, including *Curcuma domestica*, *C. xanthorrhiza*, *C. mangga*, *C. zedoaria,* and *C. aeruginosa* against multidrug-resistant *Pseudomonas aeruginosa* (MDR *P. aeruginosa*). Furthermore, the mechanism action of *Curcuma* species in combination with antibiotic against MDR *P. aeruginosa* and its chemical component were also investigated.

Methods: Determination of minimum inhibitory concentration (MIC) was carried out by the microdilution method. The synergistic effects of the extract and tetracycline were determined by the checkerboard method. The effect of the combination of *Curcuma* species and tetracycline to prevent bacterial resistance was investigated using inhibition of biofilm formation, permeability of bacterial cell membrane, and EtBr accumulation methods. Gas chromatography-mass spectrometry (GC-MS) analysis was also performed.

Results: MIC of *C. domestica*, *C. xanthorrhiza,* and *C. mangga* against MDR *P. aeruginosa* were 125, 250, and 125 µg/mL, respectively. *C. xanthorrhiza* ethanol extract (7.8 µg/mL) in combination with tetracycline (1.9 µg/mL) revealed a synergistic activity with Fractional Inhibitory Concentration Index (FICI) value of 0.06. The combination of *C. xanthorrhiza* ethanol extract and tetracycline showed inhibitory effects on biofilm formation and efflux pump of MDR *P. aeruginosa*. This combination also had bacteriolytic activity. GC-MS analysis led to the identification of ar-turmerone (11.63%) and xanthorrhizol (11.36%) as the major compounds.

Conclusion: Combination of *C. xanthorrhiza* ethanol extract and tetracycline might be developed as an alternative treatment against MDR *P. aeruginosa*.

Implication for health policy/practice/research/medical education:
The study proposes that the combination of *Curcuma xanthorrhiza* extract and tetracycline holds promise as an alternative treatment for multidrug-resistant *Pseudomonas aeruginosa* infections. This combination effectively inhibits biofilm formation and efflux pump, enhancing its efficacy against antibiotic-resistant bacteria. Additionally, it exhibits bacteriolytic activity against MDR *P. aeruginosa*, potentially contributing to the reduction of antibiotic resistance and offering a natural-based therapy. However, further research is essential to elucidate its safety and effectiveness for human use.

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Introduction

Ineffective antibiotic consumption causes global health problems. According to the Centers for Diseases Control and Prevention, antibacterial resistance has contributed to approximately 1.5 million deaths in the past five years (1). *Pseudomonas aeruginosa*, a pathogenic bacterium, exhibits phenotype of multi-drug resistance (MDR) due to genetic mutation resulting from the irrational use and prolonged consumption of antibiotics (2). This resistance extends to various conventional antibiotics, including penicillin, cephalosporins, tetracycline, chloramphenicol, and macrolides, contributing to increased mortality, morbidity, and hospital costs (3). *P. aeruginosa* is generally less susceptible to tetracycline antibiotics because tetracycline induces the type-III secretion system and consequently enhances cytotoxicity of *P. aeruginosa* (4). However, sub-inhibitory concentrations of tetracycline as protein synthesis inhibitors also induce the MexXY multidrug efflux system. The MexAB-OprM, MexCD-Oprl, and MexXY-OprM efflux systems create these bacteria to become intrinsically resistant to tetracyclines (5). *P. aeruginosa* deploys multiple resistance mechanisms, such as β-lactamase production, reduced membrane permeability, loss of outer membrane porin D2 (OprD), acquisition of plasmids/transposons, modified DNA gyrase, and the production of multi-substrate efflux pumps (6,7).

Natural compounds, including those from *Curcuma* species, are vital sources of medicinal products with significant therapeutic activity in many infectious diseases, including antibacterial properties. *Curcuma* species, well-known for antibacterial compounds like curcumin, desmethoxycurcumin, and bisdemethoxycurcumin, has demonstrated efficacy against various microorganisms, including bacteria, fungi, viruses, and parasites (8). The rhizomes of *Curcuma* species are widely used in folk medicines to treat various diseases and disorders, ranging from cough, vomiting, diarrhea, fever, abdominal pain, rheumatism, urinary tract infections, malaria infection, and hypercholesterolemia. Curcumin, a phenolic compound found in *Curcuma longa*, is recognized for its antibacterial, anti-inflammatory, anticancer, and antioxidant properties (9). Despite these known effects, the specific impacts of *Curcuma* species, particularly *Curcuma domestica*, *C. xanthorrhiza*, *C. mangga, C. zedoaria* and *C. aeruginosa* against MDR *P. aeruginosa* are rarely reported.

This present study was carried out to investigate the antibacterial effects of five *Curcuma* species (*Curcuma domestica*, *C. xanthorrhiza*, *C. mangga, C. zedoaria,* and *C. aeruginosa*) against MDR *P. aeruginosa*. Moreover, the study explored the bacteriolytic, antibiofilm, and efflux pump inhibitory properties of the most potent *Curcuma* species. Furthermore, the synergistic effect of the most potent *Curcuma* species and tetracycline was determined. The identification of the chemical components of the extract was also performed. The result of this study can be used as an approach to treat infectious diseases, especially caused by the MDR *P. aeruginosa*. Subsequently, it would help to overcome the problem of antibiotic resistance and improve the clinical outcomes of infected patients.

Materials and Methods

Material

Tetracycline (lot no. 94F-0497), ethidium bromide, phosphate buffer saline, and crystal violet were purchased from Sigma-Aldrich, UK. Brain heart infusion (BHI) (Himedia) was also used. Agar was obtained from Becton (New Jersey, USA).

Bacterial strains

Multidrug-resistant *P. aeruginosa* (strain M19) was obtained from clinical isolate collected by Marine Education and Research Organisation (MERO) Foundation, Indonesia.

Plant materials

Fresh rhizomes of five *Curcuma* species were collected from Medan, North Sumatera. The plants were identified by a botanist from Medanense (MEDA) Herbarium, Universitas Sumatera Utara, and voucher specimens of *C. domestica*, *C. xanthorrhiza*, *C. mangga, C. zedoaria,* and *C. aeruginosa* were deposited there with herbarium numbers 192/MEDA/2022, 193/MEDA/2022, 194/MEDA/2022, 195/MEDA/2022, and 196/MEDA/2022, respectively. The rhizomes were cleaned, sliced, dried, and eventually grounded into a powder.

Extraction

Powdered plant materials underwent maceration using ethanol. The resulting extract was obtained by evaporating the organic solvent using a rotary evaporator, and the yield was calculated (10).

Phytochemical screening

The ethanol extracts were subjected to preliminary phytochemical screening, including the examination of alkaloid, glycoside, flavonoid, tannin, saponin, and steroid/triterpenoid contents following standard procedures (11,12). Alkaloid screening used a Dragendorff and Mayer reagent. For Flavonoid screening we used some reagents including magnesium powder, concentrated hydrochloric acid (HCl), and amyl alcohol. For phytochemical screening of phenol group we used ferric trichloride ($FeCl_3$) 2% as a reagent and for saponin screening we only treated by shaking the solution vigorously in test tubes. For glycoside group screening we used Liebermann-Burchard and for phytochemical screening of steroid/triterpenoid group we used acetic anhydride and concentrated sulfuric acid (11,12).

Determination of minimum inhibitory concentrations (MICs)

The antibacterial activities of the ethanol extracts of *Curcuma* species were investigated by broth microdilution technique using BHI broth (13). Two-fold serial dilutions of the extract and tetracycline were prepared on 96-well microplates to obtain the concentrations of 500, 250, 125, 62.5, 31.25, 15.6, 7.8, and 3.9 µg/mL. The bacterial suspensions were diluted in 0.9% NaCl to produce 1×10^6 CFU/mL after being previously adjusted with 0.5 McFarland standard (equal to 1×10^8 CFU/mL). The microplate was incubated for 24 hours at 37 °C. The MIC value, indicating the lowest concentration preventing bacterial growth, was determined. The MIC was determined through visual observation. The lowest concentration in wells that showed clear suspension and without precipitation was conducted as the MIC value.

Antibiofilm formation assay

The antibiofilm activity of extract was carried out in a 96-wells plate according to a previous procedure with a slight modification (14). Several concentrations of BHI-containing extract (in concentration ranging from 1/4 MIC to 2 MIC) were prepared and mixed with 100 µL of the bacterial suspension in normal saline. Afterward, the microplate was incubated for 24 hours at 37 °C. The wells without treatment served as the control. After incubation, the supernatant was removed, the plate was continuously washed with PBH pH 7.3, and air-dried. Following that, 200 µL of 0.1% crystal violet stain was added to each well and incubated again at 37 °C for 30 minutes. The biofilms were then solubilized with ethanol 96% before being analyzed for absorbance. The absorbance was determined using a microplate reader (Thermo Scientific/Multiskan GO) at 560 nm.

Checkerboard assay

The checkerboard assay was conducted to examine the interaction of extract and tetracycline against MDR *P. aeruginosa* by calculating the Fractional Inhibitory Concentration Index (FICI) as previously reported (15). Briefly, a two-fold dilution with BHIB of the ethanol extract was prepared along the x-axis, and the two-fold dilution of antibiotic was prepared along the y-axis of the 96-well plate. The MDR *P. aeruginosa* suspension was then added to each well and incubated at 37 °C for a full day. The FICI value was calculated using the following formula:

$$FICI = \frac{\text{MIC of } \textit{Curcuma} \text{ extract in combination}}{\text{MIC of } \textit{Curcuma} \text{ extract alone}} + \frac{\text{MIC of tetracycline in combination}}{\text{MIC of tetracycline alone}}$$

The results reflected synergy if FICI ≤0.5, additive (0.5 ≤ FICI ≤ 1), indifferent (1 ≤ FICI ≤ 4) or antagonistic (FICI > 4 (16).

It should be noted that the phenomenon of synergistic effect is noticed when the combined administration of multiple antibacterial agents has a more pronounced therapeutic effect compared to the sum of the individual effects exerted by each constituent agent. Additive effects manifest when a combination of antibacterial drugs exhibits an effect that is equivalent to the cumulative impact of each individual constituent. The phenomenon known as the indifferent effect occurs when a mixture of antibacterial agents or a combination of an antibacterial agent and an inert substance produces an equivalent effect to that of the most potent active component. Different from the indifferent definition, the antagonistic effects in a therapy combination occur when the observed activity is lower compared to the effect of the most effective individual component (17,18).

Loss of 260-nm-absorbing material

The activity of the compound on compromising the integrity of bacterial cell membrane was conducted by measuring the loss of 260 nm-absorbing material based on a previous study with a slight modification (17). An amount of 5 mL of overnight bacterial cultures was centrifuged at 3000 rpm for 1 h, washed, and resuspended in sterile saline solution to obtain bacterial suspensions. Then, these suspensions were mixed with sample solution in Eppendorf Tube and centrifuged at 25 °C for 15 minutes (13000 rpm). The supernatant was collected for analyzing the bacteriolytic activity, while the precipitation was used to investigate the morphology of the cells under SEM analysis. The OD_{260} of the supernatant was determined to quantify the absorbance of cytoplasmic materials.

Bacteriolysis assay

Bacteriolysis assay was conducted following a previous study (19). A volume of 500 µL of 0.001% crystal violet solution was added to an Eppendorf tube, followed by centrifugation at 13000 rpm (25 °C) for 15 minutes. The supernatant was then collected and its optical density (OD) was quantified using a microplate reader (Thermo Scientific/Multiskan GO) at 590 nm. The percentage of crystal violet uptake was determined using the following formula:

$$\frac{\text{OD value of the sample}}{\text{OD value of crystal violet solution}} \times 100\%$$

Scanning electron microscope (SEM) analysis

The effects of the *Curcuma* species extracts on MDR *P. aeruginosa* cells were visualized under SEM. The bacterial pellets, obtained from the preparation in loss of 260-nm-absorbing material assay, were washed twice with 200 µL of PBS pH 7.3 and fixed with 2.5% glutaraldehyde in 0.1 M PBS. Thereafter, the bacterial cells were processed using

the biological sample preparation technology of electron microscopy. The samples were coated with platinum film before observing them under SEM. This analysis was performed by field emission SEM (FE-SEM).

EtBr efflux assay

The inhibition of efflux pump was monitored using fluorescence spectroscopy. Overnight cultures of MDR *P. aeruginosa* cells were treated with samples for 30 min. Then, 0.5 mg/L EtBr 50 µL was added and re-incubated for 5 to 30 minutes. The fluorescence was observed using a fluorescence spectroscopy with the excitation and emission wavelengths of 530 and 590 nm, respectively.

Gas chromatography-mass spectrometry (GC-MS) analysis of ethanol extract

GC-MS analysis of the ethanol extract of *Curcuma* species was performed according to a previous study with a slight modification (20). Agilent 7809B GC system coupled with 5977A MS detector was employed. Separation of compounds contained in the extract was achieved using an Agilent HP-5MS capillary column (5%-phenyl)-methylpolysiloxane phase (dimension: 30 m, ID: 0.25 mm, film: 0.25 m) and helium as the carrier gas at a constant flow rate of 1 mL/min. The temperature in GC instrument increased at a rate of 10°C/min from 40 °C to 300 °C, and the volume of each sample injection was 1 µL. The electron impact (EI) with energy of 70 eV was employed in MS detector. The mass range for the MS analysis, which included 30-600 m/z, was done in full scan mode. All peaks in the result of this analysis were tentatively identified by comparing the mass spectral data with the references in National Institute of Standard Technology (NIST) 17 library and NIST Chemistry WebBook.

Statistical analysis

Data analysis was performed by SPSS 20, involving analysis of variance (ANOVA) followed by a post hoc Tukey test. All data are presented as mean ± SD, and a significance level of $P < 0.05$ indicates statistical significance.

Results

Phytochemical contents

Table 1 shows the secondary metabolite compounds identified in the ethanol extracts of *Curcuma* species, as a result of phytochemical screening. The predominant secondary metabolite compounds found in ethanol extract included flavonoids, tannins, glycosides, steroids, and triterpenoids. Notably, secondary alkaloid metabolites were exclusively present in *C. aeruginosa* and *C. zedoaria*.

Minimum inhibitory concentrations

According to the results of antibacterial activity determination using microdilution method, the MICs of the ethanol extracts of *Curcuma species* against MDR *P. aeruginosa* were 125, 250, and 125 µg/mL for *C. domestica, C. xanthorrhiza,* and *C. mangga*, respectively. In contrast, the ethanol extracts of *C. zedoaria* and *C. aeruginosa* exhibited MIC value of >500 µg/mL. Meanwhile, tetracycline exhibited MIC value of 62.5 µg/mL. According to these results, three samples (ethanol extracts of *C. domestica, C. xanthorrhiza*, and *C. mangga*) were chosen for checkerboard assay to determine their interaction with tetracycline.

Checkerboard assay

The interaction between the *Curcuma* species extracts and tetracycline against MDR *P. aeruginosa* was assessed by calculating the FICI value. The combination which exhibited synergy activity was further examined for its mechanism of action in inhibiting resistance. Based on the result of MIC determination, the combination of *C. domestica* (125 µg/mL) and tetracycline (1.9 µg/mL) resulted in an additive interaction (FICI value of 1.03). Similarly, the combination of *C. mangga* ethanol extract (62.5 µg/mL) and tetracycline (62.5 µg/mL) exhibited an indifferent interaction against MDR *P. aeruginosa* with a FICI value of 1.5. In contrast, the combination of *C. xanthorrhiza* (7.8 µg/mL) and tetracycline (1.9 µg/mL) showed a synergistic activity with a FICI value of 0.06 (Table 2). Consequently, only *C. xanthorrhiza* ethanol extract was selected for further analysis.

Antibiofilm formation results

The inhibition of biofilm formation was assessed by the antibiofilm formation assay. Concentrations from 2 MIC, MIC, ½ KHM to ¼ MIC are presented in Figure 1. *C. xanthorrhiza* extract demonstrated significant biofilm suppression, reaching up to 90% compared to the untreated sample.

Table 1. Phytochemical screening of the ethanol extracts of *Curcuma* species

Secondary metabolites	*Curcuma domestica*	*Curcuma xanthorrhiza*	*Curcuma mangga*	*Curcuma zedoaria*	*Curcuma aeruginosa*
Alkaloid	-	-	-	+	+
Flavonoid	+	+	+	+	+
Tannin	+	+	+	+	+
Saponin	-	-	-	-	-
Glycoside	+	+	+	+	+
Triterpenoid	+	+	+	+	+

+: Present; -: Absent.

Table 2. Fractional Inhibitory Concentration Index (FICI) value of interaction between the selected *Curcuma* species ethanol extracts and tetracycline against multidrug-resistant (MDR) *Pseudomonas aeruginosa* (n=3)

Samples	MIC a (µg/mL)	MIC c (µg/mL)	FICI	Interaction
Curcuma domestica/Tetracycline	125/62.5	125/1.9	1.03	Additive
Curcuma xanthorrhiza/Tetracycline	250/62.5	7.8/1.9	0.06	Synergy
Curcuma mangga/Tetracycline	125/62.5	62.5/62.5	1.5	Indifferent

a: Alone; c: Combination; MIC: Minimum inhibitory concentration.

Loss of 260 nm absorbing material method

The loss of intracellular material from selected bacteria was observed using a microplate reader at 260 nm. As shown in Figure 2, the OD_{260} of the combination of *C. xanthorrhiza* ethanol extract (7.8 µg/mL) and tetracycline (1.9 µg/mL) was significantly higher than the single treatment of extract or tetracycline. This suggests that the combination may induce cell damage.

Bacteriolysis assay

To observe the damage to bacterial cell membranes, crystal violet solution was added to stain the membranes. *C. xanthorrhiza* extract exhibited a higher absorbance of crystal violet at 590 nm, while a single dose of tetracycline did not show a significant effect. Surprisingly, the combination of *C. xanthorrhiza* extract and tetracycline enhanced OD_{590} nm of crystal violet solution as compared to the control group ($P < 0.05$) (Figure 3).

Visualization results with SEM

SEM was performed to observe morphological changes in MDR *P. aeruginosa* after treatment. Figure 4 illustrates the enlargement breakdown of bacterial cells. Meanwhile, the untreated group with negative control (DMSO 0.5 % v/v) showed uniformity in size and distribution (Figure

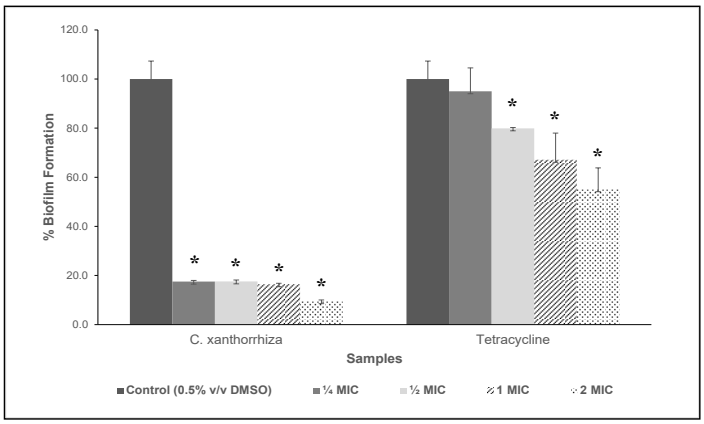

Figure 1. Effect of *Curcuma xanthorrhiza* ethanol extract and tetracycline on biofilm formation of multidrug-resistant (MDR) *Pseudomonas aeruginosa*. Data are shown as mean ± SD; n=3. **P*<0.05 compared to the respective control/DMSO.

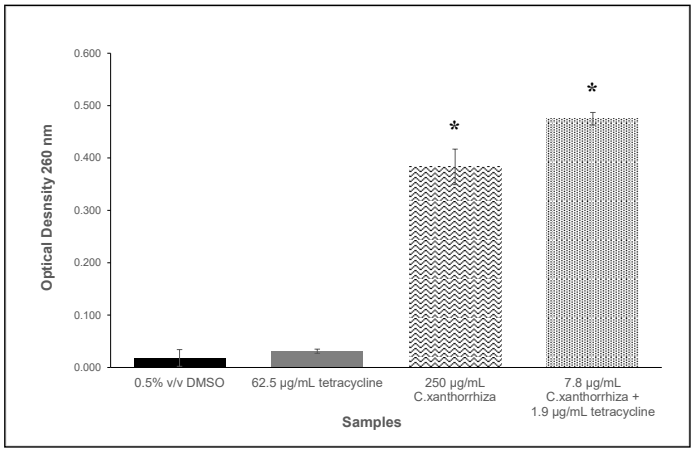

Figure 2. Effect of the combination of *Curcuma xanthorrhiza* ethanol extract and tetracycline on the loss of 260-nm-absorbing materials of multidrug-resistant (MDR) *Pseudomonas aeruginosa*. Data are shown as mean ± SD; n=3. **P*<0.05 compared to the respective control/DMSO.

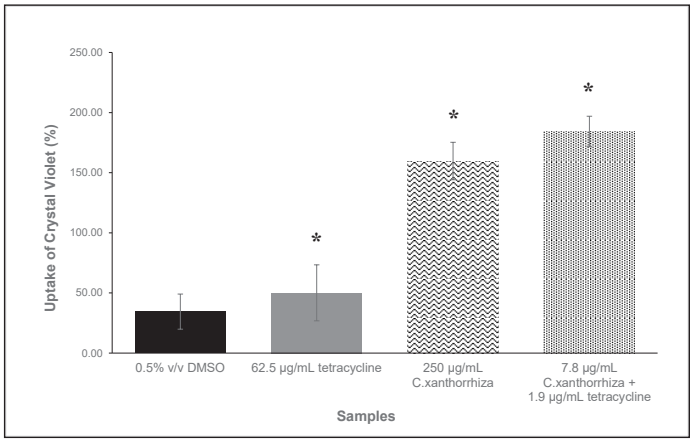

Figure 3. Effect of the combination of *Curcuma xanthorrhiza* ethanol extract and tetracycline on bacteriolysis activity of multidrug-resistant (MDR) *Pseudomonas aeruginosa*. Data are shown as mean ± SD; n=3. *P < 0.05 compared to the respective control/DMSO.

4a), and visualization under SEM showed no significant alteration in morphology of MDR *P. aeruginosa* treated with tetracycline (62.5 µg/mL). This result indicated that tetracycline had no effect on the bacteria cell morphology (Figure 4b). In contrast, MDR *P. aeruginosa* treated with *C. xanthorrhiza* (250 µg/mL) showed morphological changes. *C. xanthorrhiza* extract demonstrated holes on membrane cell (Figure 4c), and significant changes of bacterial morphology were observed in samples treated with the combination of *C. xanthorrhiza* (7.8 µg/mL) and tetracyclines (1.9 µg/mL) (Figure 4d). These results were in agreement with the assay of loss of 260-nm-absorbing materials that showed significant differences between the combination of extract and tetracycline compared with those in the control group.

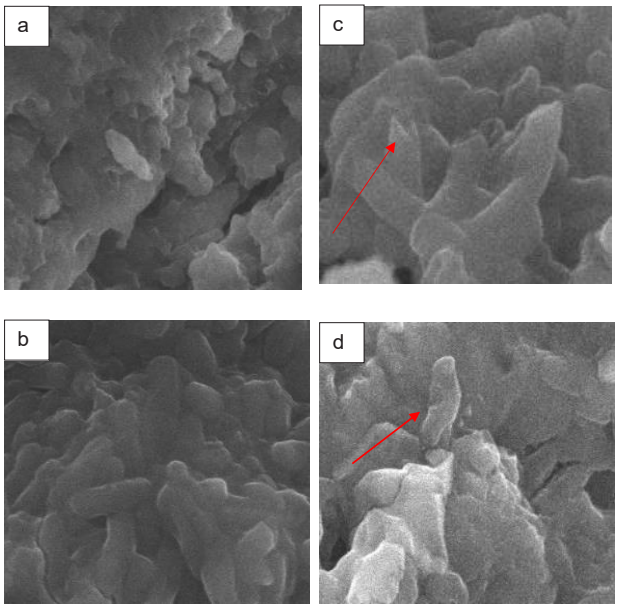

Figure 4. Visualization morphology of multidrug-resistant (MDR) *Pseudomonas aeruginosa* bacterial cells under SEM analysis after treatment with (a) 0.5% v/v DMSO; (b) 62.5 µg/mL tetracycline; (c) 250 µg/mL *Curcuma xanthorrhiza*; (d) 7.8 µg/mL *C. xanthorrhiza* + 1.9 µg/mL tetracycline. The red arrows indicate pores formation in membrane of MDR *P. aeruginosa*. MDR: Multidrug-resistant, SEM: Standard error of the mean, DMSO: Dimethyl sulfoxide.

Accumulation of EtBr

In this analysis, the increase in intracellular EtBr accumulation was performed to investigate the activity of sample as efflux pump inhibitor in MDR *P. aeruginosa*, measured by the fluorometric method at 0, 5, and 15 minutes. The combination of *C. xanthorrhiza* (7.8 µg/mL) and tetracycline (1.9 µg/mL) showed a higher accumulation, which was significantly different from the other treated samples (Figure 5).

GC-MS analysis of ethanol extract

As shown in Table 3, the compounds identified in the GC-MS analysis of *C. xanthorrhiza* extracts were 56 compounds. The major compounds present in *C. xanthorrhiza* were ar-turmerone (11.63%) and xanthorrhizol (11.36%).

Discussion

Phytochemical screening of *Curcuma* species extract showed the presence of various secondary metabolites. The secondary metabolites present in *Curcuma* species extract have exhibited antibacterial activities. Alkaloids, for instance, possess antibacterial properties by interfering with peptidoglycan components in bacterial cells, leading to cell death. Terpenoids, a group with potential antimicrobial activity, have demonstrated antifungal, antibacterial, and antiviral properties. The mechanisms of action as an antibacterial are supposed to work by damaging the bacterial cell walls by interfering with the peptidoglycan components of bacteria cells. Flavonoids eradicate bacteria by disrupting the integrity of bacterial cell membranes. Tungmunnithum et al described flavonoids as phenolic compounds with a protein-coagulating properties (21). According to Farha et al, tannins, identified as polyphenol compounds, exhibited

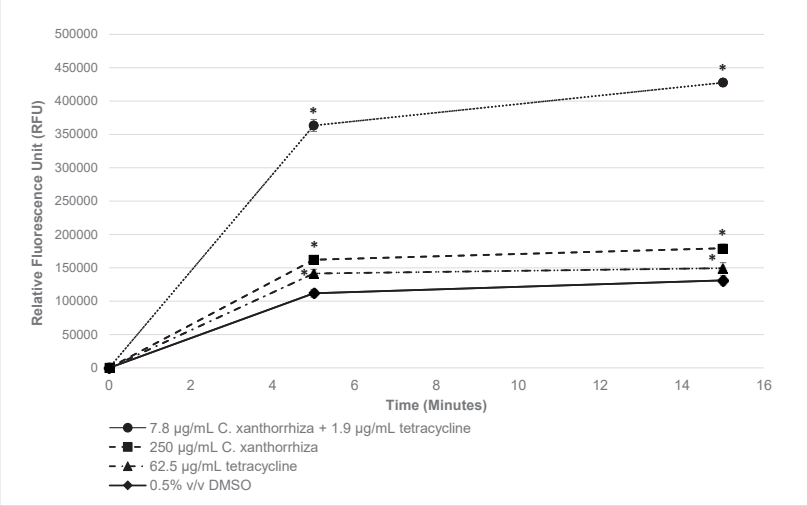

Figure 5. The effect of the combination of *Curcuma xanthorrhiza* extract and tetracycline on efflux pump of multidrug-resistant (MDR) *Pseudomonas aeruginosa*. Data are shown as mean ± SD; n=3. **P* < 0.05 compared to the respective control.

antibacterial activity by interfering with bacterial cell walls or membranes, impacting membrane permeability and hindering metabolic activities (22). Tannins also precipitate proteins, sharing similar effects to phenolic compounds. The ability of antibacterial compounds to inhibit bacterial growth is influenced by the stability of proteins, lipids, salts, and acidity (pH) levels in the growth medium. The non-polar mucopeptide or peptidoglycan layer in the gram-positive bacterial cell composition facilitates the penetration of lipophilic compounds, leading to interactions with proteins and peptidoglycans layers, causing structural damage eventual bacterial death (23).

The broth microdilution method was employed to evaluate the antibacterial activity of five Curcuma sp. ethanol extract, including *C. domestica* Val., *C. xanthorrhiza, C. mangga, C. zedoaria* and *C. aeruginosa* against MDR *P. aeruginosa*. While *C. domestica* and *C. mangga* exhibited the lowest MIC of 125 µg/mL, only the combination of *C. xanthorrhiza* (7.8 µg/mL) and tetracycline (1.9 µg/mL) demonstrated a synergistic interaction with FICI value of 0.06. This finding indicated that the combination of *C. xanthorrhiza* extract and tetracycline significantly enhanced the antibacterial effect of tetracycline by 64-fold reduction. This result aligns with previous study reporting strong antibacterial activity of essential oil from *C. xanthorrhiza* against selected clinical isolates (24). Biofilms, crucial in *P. aeruginosa*-induced infections, contribute to resistance against antibacterial agents, making their inhibition as a critical therapeutic strategy. Some of these resistances cause various nosocomial infections and life-threatening diseases and they are the most major cause of chronic lung infections. Bacteria can produce agents called 'virulence traits', enabling them to counteract the host immune system responses, as well as colonizing and producing toxins. Biofilms also indicate a population of planktonic bacterial cells that can attach to various surfaces, both abiotic and biotic surfaces, consisting of polysaccharides, DNA, proteins, and other components. Bacterial cells that are covered by biofilms are often more resistant to antibacterial agents (thousands of times) than planktonic cells. Therefore, biofilms are considered one of the reasons for the difficulty of treatment with antibiotics (25). The ethanol extract of *C. xanthorrhiza* exhibited dose-dependent antibiofilm activity, with higher concentrations displaying greater efficacy in inhibiting biofilm formation in MDR *P. aeruginosa*. This result was also supported by previous studies reporting the effect of turmeric and red ginger essential oil as antibiofilm agent against *Streptococcus* mutants and *Candida albicans, respectively* (26,27). Phenolic compounds, such as those present in *C. xanthorrhiza*, have been linked to decreased production of pyocyanin and the swarming motility of *P. aeruginosa* clinical isolates (28).

There are various mechanisms of antibiotic resistance, including intrinsic, adaptive, and acquired mechanism. The intrinsic mechanism of resistance in *P. aeruginosa* include decreasing the plasma membrane permeability, producing antibiotic efflux pumps and β-lactamases, which metabolize β-lactam antibiotics (29). The combination of *C. xanthorrhiza* extract and tetracycline showed the highest OD_{260} value, definitely indicating more intracellular component leakage from bacterial cells (260 nm), likely due to the increased membrane permeability caused by secondary metabolites contained in *C. xanthorrhiza*. The treated sample with the combination of extract and tetracycline had a significant difference compared to other groups (*P* value < 0.05). The combination of extract and tetracycline displayed massive effects on damaging the morphology of bacteria cells. Previous studies support these findings, reporting that the curcumin compound in

Table 3. Gas chromatography–mass spectrometry (GC-MS) analysis of *Curcuma xanthorrhiza* ethanol extract

No.	Compounds	Percent	Retention time (min)
Monoterpenes			
1	1,3,8-p-Menthatriene	2.46	16.174
2	Durene	1.22	17.825
Oxygenated monoterpenes			
1	trans-α-Decalone	0.71	18.039
2	α-Thujol		
3	2-Nonen-4-one, 2-methyl-	1.44	20.143
4	2-Isopropylidene-3-methylhexa-3,5-dienal	0.09	22.122
Sesquiterpenes			
1	α-Curcumene	2.66	14.447
2	Valerena-4,7(11)-diene	0.44	14.636
3	α-Guaiene		
4	Intermedeol		
5	α-Selinene	0.34	14.712
6	α-Gurgujene		
7	β-Curcumene	2.08	14.8
8	Isoitalicene		
9	δ-Cadinene	0.55	14.938
10	β-Sesquiphellandrene	1.00	14.989
11	Caryophyllene	0.14	15.19
12	α-Yalangene		
13	Isodaucene		
14	α-Curcumene	0.28	15.241
15	Alloaromadendrene		
16	α-Himachalene		
17	Thujopsene	1.94	15.354
18	Dihydrocurcumene	1.09	15.657
19	Virdiflorene	0.39	15.858
20	Longifolene		
21	α-Acoradiene	0.92	16.287
22	α-Muurolene	1.32	16.476
23	Cyclosativene		
24	γ-Elemene	0.09	22.122
25	β-Germacrene	1.74	19.412
26	β-Caryophellene	0.58	19.173
27	β-Cyclogermacrane	0.47	18.808
28	β-Germacrene		
Oxygenated sesquiterpenes			
1	Humulenol-II	1.94	15.354
2	Sesquisabinene hydrate		
3	Sesquisabinene hydrate	0.91	15.808
4	β-Acorenol		
5	β-Elemenone	4.44	15.934
6	Sesquisabinene hydrate	1.00	16.085
7	Zingiberenol		
8	Zingiberenol	0.92	
9	Isospathulenol		16.287
10	T-murulol	1.32	16.476
11	Tumerone	5.53	16.728
12	γ-Atlantone (Z)		
13	aR-Turmerone	11.63	16.69
14	Curlone	9.14	17.106
15	Curcuphenol	1.01	17.207
16	α-Bisabolene oxide	0.53	17.434
17	Cedren-13-ol, 8-		
18	γ-Atlantone (Z)	1.56	16.993
19	Xanthorrizol	11.36	17.585
20	γ-Atlantone (Z)	2.61	17.938
21	β-Spathulenol	0.40	18.215
22	β-Elemenone		
23	Acorenone B	0.60	18.266
24	α-Bisabolene oxide		
25	Diepicedrene-1-oxide	0.58	18.429
26	Humulenol	1.51	18.682
27	Isoaromadendrene epoxide		
28	α-Bisabolene oxide	0.80	18.871
29	Acorenone B		
30	Cryptomerione		
31	Isoaromadendrene epoxide	0.47	18.971
32	α-Cyperone	0.58	19.173
33	α-Oxobisabolene	1.09	19.274
34	aR-Turmerone	0.38	19.98
35	α-Bisabolene oxide	0.41	20.093
36	aR-Turmerone		
37	γ-Atlantone (Z)	0.28	21.000
38	α-Bisabolene oxide	0.09	22.122
39	Nootkatone	0.11	22.714

C. xanthorrhiza restrains cytokinesis and multiplication of *Staphylococcus aureus*, and disrupts the bacterial cell wall and membrane, leading to cell lysis (30). The ability of *C. xanthorrhiza* to increase the permeability of the cell membrane of MDR *P. aeruginosa* or disrupt the integrity of the cell wall and bacterial cell membrane suggest combining *C. xanthorrhiza* extract with tetracycline. The combination of *C. xanthorrhiza* extract and tetracycline has the potential to inhibit the efflux pump, demonstrated by an increase in the Relative Fluorescence Unit of EtBr. This finding was supported by a previous study by Seukep et al, that reported Zingiberaceae as an excellent source of efflux pump inhibitors (31). Curcumin, present in *Curcuma longa* species has been reported to inhibit the NorA, MdeA, TetK, and MsrA efflux pump of *Staphylococcus aureus*, and the *P. aeruginosa* efflux pumps. Thus, this extract can be used in combination with antibiotics to treat the infectious diseases.

The major compounds present in *C. xanthorrhiza* were ar-turmerone (11.63%) and xanthorrhizol (11.36%). A previous study also reported the presence of active compounds like xanthorrhizol, curcumin, and other volatile compounds in *C. xanthorrhiza* (32).

Sesquiterpenes, particularly ar-turmerone and xanthorrhizol, possess antimicrobial properties, impacting essential bacterial survival processes. Sesquiterpene compounds are known to affect bacterial mechanisms involved in oxygen absorption for respiration (33).

Based on GC-MS analysis, *C. xanthorrhiza* also have several phenolic compounds such as xanthorrhizol as the main component. Xanthorrhizol has antimicrobial

properties, such as anticandidal, antifungal, and antibacterial effects. Xanthorrhizol presented the antibacterial activity against *Streptococcus* species, strongly inhibited Methicillin-resistant *Staphylococcus aureus*, *Escherichia coli*, and *Propionibacterium acnes* (34). The strong antibacterial activity of the extract might be due to the presence of high amounts of ar-turmerone (11.63%) and xanthorrhizol although other constituents may also contribute.

According to the results obtained, the combination of ethanol extract of *C. xanthorrhiza* rhizomes and tetracycline has the ability to inhibit the growth of MDR *P. aeruginosa* through several modes of action, including inhibition of biofilm formation, the ability to degrade bacterial cell membranes, and the inhibition of the efflux pump of MDR *P. aeruginosa*. However, the efficacy and safety of this combination to eradicate MDR *P. aeruginosa* in human body still could not be confirmed. In vivo preclinical study should be performed to evaluate the safety and effectiveness. Nevertheless, this study can be used as an approach to treat infectious diseases, especially caused by MDR *P. aeruginosa*. These approaches were expected to overcome the problem of antibiotic resistance and improve the clinical outcomes of infected patients. Therefore, it is hoped that this research will provide initial information that will be continued to a clinical trial to complete the data of their toxicity and develop into pharmaceutical dosage forms that are safe to consume.

Conclusions

The ethanol extracts of *Curcuma species*, including *C. domestica*, *C. xanthorrhiza* and *C. mangga,* exhibited strong antibacterial activity against MDR *P. aeruginosa*. Additionally, the combination of *C. xanthorrhiza* ethanol extract and tetracycline demonstrated a synergistic interaction against MDR *P. aeruginosa*. This extract also exhibited antibiofilm formation compared to the negative control. The combination of extract and tetracycline changed the permeability of membrane cell, inducing cell lysis and inhibiting efflux pump. aR-Turmerone and xanthorrhizol were the most abundant compounds present in *C. xanthorrhiza*. The results suggest that the natural active compounds in *C. xanthorrhiza* ethanol extract may have specific targets in MDR *P. aeruginosa*, offering alternative strategy to suppress antibiotic resistance. However, further studies are required to elaborate on its efficacy and safety in whole animals.

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Conflicts of interests

All authors of this research declare no conflict of interest.

Ethical considerations

Authors have carefully monitored ethical issues such as text plagiarism, duplicated publication, misconduct, data fabrication, and falsification.

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